Moss et al. Int J Trop Dis 2018, 1:007
Volume 1 | Issue 1
Open Access

International Journal of
Tropical Diseases

RESEARCH ARTICLE

Serological Evidence of Dengue and Chikungunya Exposures in Malian Children by Multiplex Bead Assay

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Abstract

We opportunistically included a chikungunya envelope 1 recombinant virus antigen (CHIKV E1), a dengue 2 virus-like protein (DENV-2 VLP), and a DENV-3 VLP in a multiplex bead assay (MBA) for serological assessment involving improvements in water, sanitation, and hygiene for students attending 42 elementary schools in southern Mali in Western Africa. From students attending grades 1-6,805 dried blood spots (DBS) were collected. Immunoglobulin (Ig) G was eluted from the DBS and was probed for responses to the antigens. For all students, positive IgG responses to CHIKV E1, DENV-2 VLP, and DENV-3 VLP were 6.2%, 24.3%, and 14.8%, respectively. However, in some schools the positivity rate was as high as 21.1% for CHIKV E1 and 66.7% for both DENV-2 and DENV-3 VLP. In one set of schools, cluster analysis from SaTScan software showed a relative risk of 3.1 and 5.1 for DENV-2 and DENV-3 VLP, respectively, compared with schools outside the cluster. The MBA serology platform is very flexible in that additional antigens other than the intended targets can be included with very little cost and can provide additional effective opportunities for evaluation of public health activities.

Keywords

Multiplex bead assay, Serology, Chikungunya, Dengue, Virus-like protein

Introduction

It has been estimated that 3.9 billion people in 128 countries are at risk of DENV infection and that about 390 million DENV infections occur every year of which 96 million manifest clinical symptoms [1,2]. More than 2.38 million DENV cases with 1032 deaths were reported in the Americas in 2016, and Brazil alone contributed about 1.5 million cases [3]. DENV has four serotypes and belongs to the genus *Flavivirus*, family *Flaviviridae*, and can lead to dengue fever or dengue hemorrhagic fever. CHIKV was first isolated in the 1952 outbreak in Tanzania, and limited CHIKV outbreaks occurred in Asia in the 50s and 60s, and then spread throughout Africa and Europe in the early 2000s [4,5]. In 2013, CHIKV cases were reported in St. Martin Island and were known to have

Citation: Moss DM, Whitney MT, Chard AN, Trinies V, Doumbia S, et al. (2018) Serological Evidence of Dengue and Chikungunya Exposures in Malian Children by Multiplex Bead Assay. Int J Trop Dis 1:007.

Accepted: October 09, 2018; **Published:** October 11, 2018

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spread throughout the Caribbean, Central America, and South America [6]. From 2006-2014, CHIKV has caused outbreaks in many islands in the Indian and southern Pacific oceans, and currently, of all confirmed CHIKV cases in the Americas, Brazil accounts for more than 90% [7,8]. CHIKV belongs to the genus *Alphavirus*, family *Togaviridae*. Both viruses can cause fever, arthralgia, malaise, headache, and rash, and are transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitos. There is no treatment for these pathogens other than nutrition and hydration. Because vaccines are not yet perfected [9,10], protection of the human body from mosquitos and mosquito control in the environment are the only defense.

CHIKV and DENV are known to be endemic in Mali in Western Africa, but information on prevalence is limited. In 2006, one study showed 87 of 93 febrile patients were immunoglobulin (Ig) G positive to DENV by an enzyme-linked immunosorbent assay (ELISA) [11]. Another study conducted annually from 2009-2013, among 376 returning expatriates from Germany with fever showed IgG prevalence ranging from 2.8-8.4% for CHIKV and 31.0-43.0% for DENV [12].

Here, a multiplex bead assay (MBA) was used in a water, sanitation, and hygiene (WASH) study to determine serological differences to various antigens between elementary students receiving and not receiving WASH intervention [13,14]. On this same study with the same DBS, other MBA reports are available [15-18]. The MBA, utilizing DBS, was selected due to its proven performance and cost effectiveness [19-22]. Because very little information is available on prevalence of CHIKV and DENV in Mali, we capitalized on this WASH study by adding CHIKV and DENV antigens to the array of antigens from enteric pathogens, neglected tropical diseases, and vaccine-preventable diseases. Dried blood spots (DBS) were collected from the students, and the eluted IgG was assessed on all antigens simultaneously using the MBA.

Materials and Methods

Study population

Using stratified random sampling based on region, 21 primary schools with WASH improvements and 21 matched-comparison schools without WASH improvements were selected from a set of 200 schools participating in a longitudinal matched-control evaluation of a WASH in schools intervention, for a total of 42 schools. Matched-comparison schools were located within the same educational district and matched on baseline enrollment size and school WASH characteristics [14]. An average of 19 students (range, 14-20) per school were selected from a list of all pupils enrolled in elementary classes 1-6 using stratified random sampling based on pupil sex and grade. The study consisted of 805 students, age 4-17 years (58% provided age), in the 42 elementary schools in the regions of Mopti, Sikasso, Koulikoro, and the capital of Bamako.This sample size was determined by the maximum number of samples that could be collected based on study budget. School enrollments ranged from 71-651 students per school, thus, the percentage of students tested per school ranged from 3.1-28.2%.

The study was reviewed and approved by the Ethics Committee of the National Institute of Public Health Research in Mali and the Institutional Review Board of Emory University (Atlanta, GA). Laboratory staff from the Centers for Disease Control and Prevention had no contact with students nor access to personal identifiers. Signed/fingerprinted informed consent was provided by parent or guardian of each randomly selected pupil prior to blood spot collection, and each pupil provided informed verbal assent. The trial was registered at Clinical Trials.gov (NCT01787058).

Blood sample collection

By finger prick, 10 µL of whole blood was collected onto each of six circular filter paper extensions (Trop Bio Pty Ltd, Townsville, Queensland, Australia). After collection, the dried blood spots (DBS) were stored at -20 °C, as previously described [20]. One DBS wheel per child was collected between January and June of 2014 during the dry season in Mali.

Antigen coupling to beads

Carboxyl groups on the surface of spectrally classified-magnetic polystyrene microspheres (MagPlex Beads; Luminex Corporation, Austin, TX) were converted to reactive esters using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide method (Calbiochem, Woburn, MA). Recombinant CHIKV E1 (CTK Biotech, San Diego, CA, wild type) was used. In addition, DENV-2 virus-like particles (VLP) and DENV-3 VLP were purified from COS-1 cell lines that constitutively expressed the premembrane/membrane and envelope proteins for DENV-2 and DENV-3, respectively. Although both DENV-2 and DENV-3 VLPs share epitopes found in the four DENV serotypes, the DENV-2 VLP has many epitopes for dengue 2 and 4 serotypes, more than DENV-3 VLP, and the DENV-3 VLP has many epitopes for dengue 3 and 1 serotypes, more than DENV-2 [23,24]. CHIKV E1, DENV-2 VLP, and DENV-3 VLP were each covalently linked to differently classified microspheres (12.5 million each) by covalent amide bonds in PBS, pH 7.2, using 8.7, 52.3, and 34.8 µg, respectively [21]. Coupling efficiency was determined using sera highly reactive to the antigens.

MBA data acquisition

Antibodies from one DBS were eluted using 500 µL PBS containing 0.5% bovine serum albumin, 0.3% Tween 20, 0.1% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinyl pyrrolidone, and 0.1% casein. Based on an estimated hematocrit of 50% in the DBS, this was a

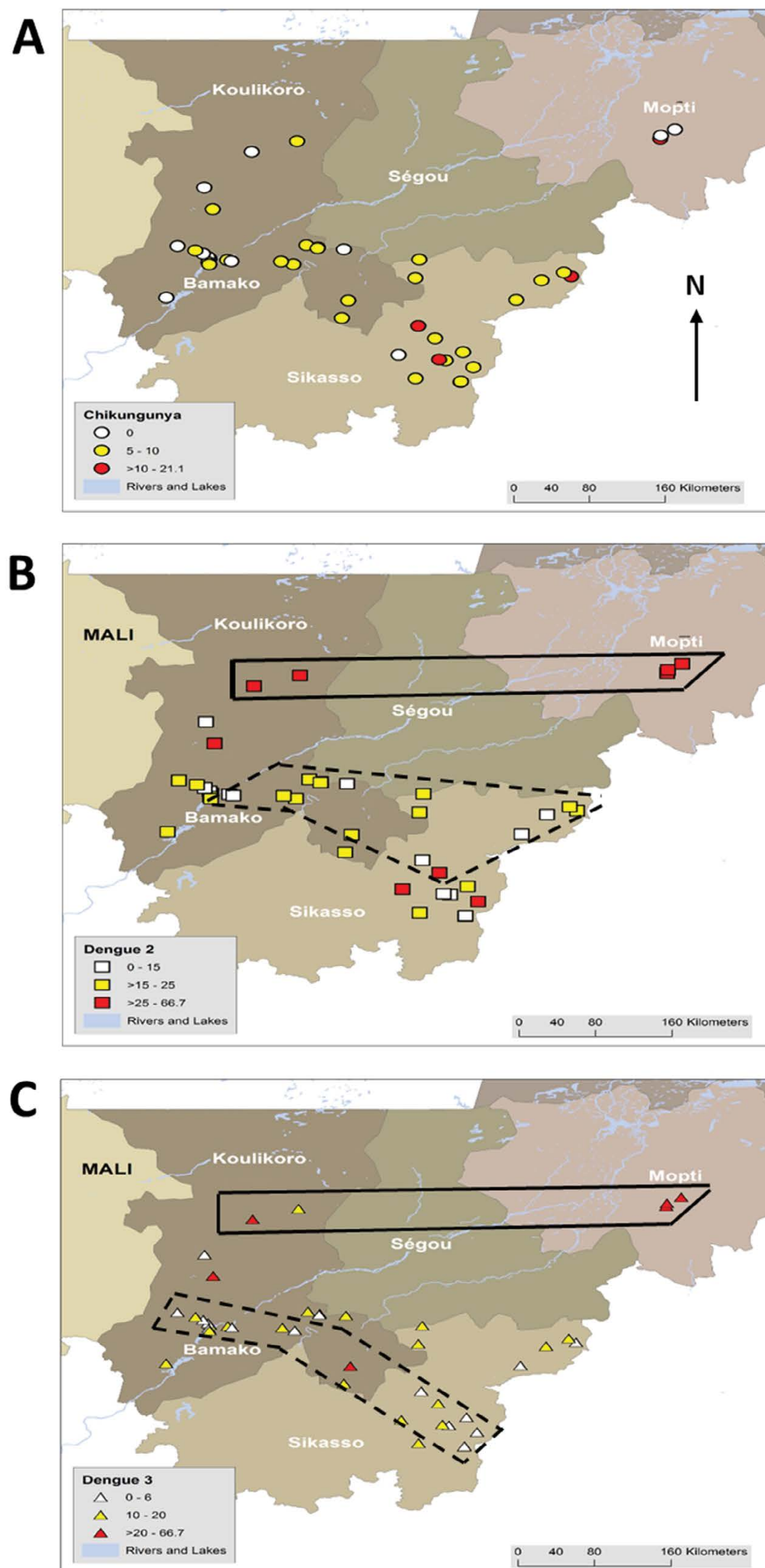

Figure 1: Map of Southern Mali in Western Africa, showing political districts and percentage positive to antigens per school.

A) Study populations consisted of elementary schools, grades 1-6 located in Mopti, n = 4; Sikasso, n = 16; Koulikoro, n = 20; and capital city district of Bamako, n = 2. Each school averaged 19 students tested with a range of 14-20. Percentage of positive IgG responses per school (colored circles) to CHIKV E1 antigen with arbitrarily selected sub-ranges of percentage positives per school: white, 0% (n = 12 schools); yellow, 5-10% (n = 12); and red, > 10-25% (n = 5); B) Same schools with percentage of positive IgG responses per school (colored squares) to DENV-2 VLP antigen with arbitrarily selected sub-ranges of percentage positives per school: white, 0-15% (n = 14 schools); yellow, > 15-25% (n = 17); and red, > 25-66.7% (n = 11); C) Same schools with percentage of positive IgG responses per school (colored triangles) to DENV-3 VLP antigen with arbitrarily selected sub-ranges of percentage positives per school: white, 0-5% (n = 16 schools); yellow, 10-20% (n = 19); and red, > 20-66.7% (n = 7). Some school symbols overlap.

1:100 serum dilution. After overnight elution at 4 °C, an additional dilution of 1:4 (1:400 serum dilution) using the same buffer but containing a final 0.3% crude and unclarified *Escherichia coli* extract (for absorption of *E. coli* antibodies for coupled antigens expressed in *E.coli*) was performed, stored overnight at 6 °C, and then exposed to antigen-coupled beads for 1.5 h at room temperature. Bound antigen-specific IgG was detected with biotinylated mouse anti-human IgG for 45 minutes followed by a 30 minute exposure to a reporter molecule, r-phycoerythrin linked with streptavidin, as described [18]. Between steps, beads were washed three times with 0.05% Tween 20 in PBS using a Bio-PlexPro II Wash Station (Bio-Rad, Hercules, CA). Data was acquired with a Bio-Plex 100 reader with Bio-Plex Manager 6.1 software (Bio-Rad) that calculated the median fluorescence intensity from the reporter molecule (MFI, based on signal intensity placed in a subset of channels 1-32,766) from each bead classification from each well and determined the mean MFI from duplicate wells. Background (bg) fluorescence from a blank with no DBS was subtracted (MFI-bg) and the difference used as data. The signal intensity, MFI-bg, is directly proportional to the amount of IgG attached to the coupled beads.

Cutoff determination

Cutoffs were determined using 86 serum specimens from North American adults, non-travelers internationally. Two outliers with responses greater than 5 standard deviations (SD) above the mean were eliminated for cutoff determinations for CHIKV E1 and DENV-3 VLP, and the remainder were used to calculate the mean plus three SD. None were eliminated for DENV-2 VLP.

MBA Validation for DENV-2 and DENV-3 VLP antigens

An ELISA, the standard CDC method for IgG detection to DENV,was used to test 60 serum samples (the ELISA not yet adapted for DBS), 30 from DENV endemic-Cambodia and 30 from DENV non-endemic North America (adults who claimed not to have traveled outside the United States). The IgG capture ELISA used a mixture of DENV-1, DENV-2, DENV-3 and DENV-4 VLP antigens,and all were propagated in COS-1 cells. The VLP were captured with a monoclonal antibody [25]. Any IgG from test serum bound to the VLP was probed with goat anti-human IgG linked to alkaline phosphatase. Color was developed using disodium p-nitrophenyl phosphate and read at 405 nm [25].

School locations

For each school, a global-positioning-system acquired latitude and longitude coordinates (https://opendatakit.org/), and ArcGIS 10.3.1(http://www.esri.com/) (Esri, Redlands, CA) were used to plot coordinates. The percentage of tested students who were IgG positive per school was plotted for each antigen, and a colored symbol, representing the percentage positive, is shown in each legend (Figure 1A, Figure 1B, and Figure 1C).

Cluster analysis

The spatial scan statistic implemented in SaTScan software was used to search for spatial clusters of schools with elevated prevalence, the number of tested students who were positive at each school [26,27]. An elliptical cluster shape was assumed and statistically significant clusters ($p < 0.05$ used for significance) are represented by the border of a convex hull of schools deemed to be inside the cluster, and the software determined the relative risk (RR) of exposure to CHIKV, DENV-2, and DENV-3.

Statistics

Correlation coefficients of the MFI-bg (IgG responses) between any combination of the CHIKV E1, DENV-2 VLP, and DENV-3 VLP antigens and other antigens was obtained by Spearman rank order correlation. Compari-

Table 1: Arbitrarily selected sub-ranges of percentage positives for the schools for each antigen with colored-coded symbols. The number of participating schools per color code, the number of students tested, the number of students positive, and the range of number of students enrolled per school.

	Antigen	**Percentage of students IgG positive per school**	**Color Scale (Symbol, Figure 1)**	**Number of Schools**	**Number of Students Tested**	**Number of Students Tested IgG Positive**	**Range of the Number of Students Enrolled per School**
	CHIKV E1	0%	White Circle, Figure 1A	12	223	0	71 - 528
	CHIKV E1	> 0-10%	Yellow Circle, Figure 1A	25	485	33	73 - 651
	CHIKV E1	15-20%	Red Circle, Figure 1A	5	97	17	131 - 388
Total				**42**	**805**	**50**	
	DENV-2 VLP	0-10%	White Square, Figure 1B	14	272	27	105 - 651
	DENV-2 VLP	> 10-25%	Yellow Square, Figure 1B	17	330	71	71 - 528
	DENV-2 VLP	> 25-66.7%	Red Square, Figure 1B	11	203	98	87 - 405
Total				**42**	**805**	**196**	
	DENV-3 VLP	0-5%	White Triangle, Figure 1C	16	301	13	105 - 596
	DENV-3 VLP	10-20%	White Triangle, Figure 1C	19	374	47	71 - 651
	DENV-3 VLP	> 20-66.7%	White Triangle, Figure 1C	7	130	59	73 - 405
Total				**42**	**805**	**119**	

sons of median MFI-bg (IgG responses) among all grade levels was performed by the Kruskal-Wallis One Way Analysis of Variance on Ranks, all pairwise multiple comparison, Dunn's method. Significance was set at $p < 0.05$.

Results

Antigen coupling and cutoffs

All coupled antigens showed high MFI-bg to sera known to be highly reactive to the antigens, indicating sufficient antigen coupling to the beads. The MFI-bg cutoffs for CHIKV E1, DENV-2 VLP, and DENV-3 VLP were 509, 558, and 632, respectively. For all students, the MFI-bg ranges (medians) were 11-27,250 (136), 9-7,620 (206), and 5-10,908 (215), respectively.

Percentage positive of those tested per school

Because the percentage of students who tested positive for each school ranged widely for each antigen, we arbitrarily selected sub-ranges of percentage positives for the schools for each antigen and assigned color-coded symbols. This is shown in Table 1 along with antigen, the number of schools in each color code, the number of students tested, the number of students who were positive, and the range of the number of students enrolled in the schools in each range.

Validation

Shown in Figure 2A are the test comparisons for binary IgG positive/IgG negative data using the CDC standard IgG capture ELISA on 60 blood samples, 30 serum samples from endemic DENV Cambodia, and 30 serum samples from non-endemic DENV North American. The ELISA showed that the DENV-2 VLP and DENV-3 VLP used in the MBA had an estimated 97% for both sensitivity and specificity [25].

Correlations

In a previous study, we showed weak correlation- of IgG responses between CHIKV E1 and either of the DENV-2 VLP and DENV-3 VLP antigens, and a relatively strong correlation between the DENV-2 VLP and DENV-3 VLP antigens [21]. Here, in agreement, a scatter plot of CHIKV E1 and DENV-2 VLP (Figure 2B) showed a weak correlation coefficient ($r^2 = 0.39$, $p < 0.001$) and a relatively strong correlation coefficient ($r^2 = 0.81$, $p < 0.001$) between DENV-2 VLP and DENV-3 VLP (Figure 2C). Weak correlation coefficients were observed in IgG responses among CHIKV E1, DENV-2 VLP, and DENV-3 VLP antigens when compared with malaria and norovirus antigens (data not shown).

GPS-located schools and percentage positive

For all students tested, the IgG prevalence was 6.2, 24.3, and 14.8% for CHIKV E1, DENV-2 VLP, and DENV-3 VLP, respectively. However, in some schools, the percentage of IgG positivity was as high as 21.1, 66.7, and 66.7%, respectively, indicating high transmission in some areas. Shown in Figure 1A, Figure 1B, and Figure 1C is a map of southern Mali and the GPS-located schools studied: Mopti, 4 schools; Sikasso, 16; Koulikoro, 20; and the Bamako capital, 2. In Figure 1A, the percentage of IgG positives to the CHIKV E1 per school (colored circles) are shown for each percentage range: white, 0% (n = 12 schools); yellow, 5-10%, (n = 25); and red, > 10-21.1%, (n = 5). Of the five reds, Mopti has one, and Sikasso four, all near the Burkina Faso/Mali border. In Figure 1B, the percentage of IgG positives to the DENV-2 VLP per school (colored squares) are shown for each percentage range: white, 0-15% (n = 14 schools); yellow, > 15-25% (n = 17); and red, > 25-66.7% (n = 11). Of the eleven reds, Koulikoro has three, Mopti four, and Sikasso four, most near the Burkina Faso/Mali border. In Figure 1C, the percentage of DENV-3 VLP IgG positives by school (colored triangles) is shown for each percentage range: white, 0-5% (n = 16 schools); yellow, 10-20% (n = 19); and red, > 20-66.7% (n = 7). Of the seven schools with high prevalence (reds), Koulikoro has three and Mopti has four. The four in Mopti and two of the three in Koulikoro are the same school locations as the red symbols for DENV-2 VLP (Figure 1B). Some school symbols overlap.

Cluster analysis

For CHIKV E1, no significant cluster of antibody positivity was found (Figure 1A). However, for DENV-2 VLP and DENV-3 VLP, a cluster of widely separated schools, four in Mopti and two in Koulikoro, were found to have a RR of 3.1 ($p < 0.001$) and 5.1 ($p < 0.001$), respectively (six schools inside solid black lines, Figure 1B and Figure 1C, respectively). Secondary but significant sets of clusters with lower risk of exposure were found for the DENV-2 VLP and DENV-3 VLP that showed RR of 0.47 ($p = 0.001$) and 0.39 ($p = 0.003$), respectively (20 schools inside dashed black lines Figure 1B and 21 schools inside dashed black lines, Figure 1C, respectively). Many yellow and red symbols were found near the Burkina Faso/Mali border, but none were identified by SaTScan software as significant.

Age verses IgG responses

The median MFI-bg for all three antigens showed an increasing trend from grades 1-6 (median ages: 6.5, 8.0, 10.0, 11.0, 12.0, and 13.0 years, respectively) and was significant ($p < 0.038$) for DENV-2 and CHIKV and approached significance for DENV-3 ($p = 0.051$), indicating an increased chance of exposure with increased age.

Discussion

This study was not optimally designed for CHIKV and DENV sero surveillance but does provide useful information. Here, the children were studied primarily for WASH intervention, and because of their youth, they have less cumulative exposures to pathogens than adults. It has been shown that IgG responses to CHIKV last for years [28], and IgG to DENV appears to be long lasting [29].

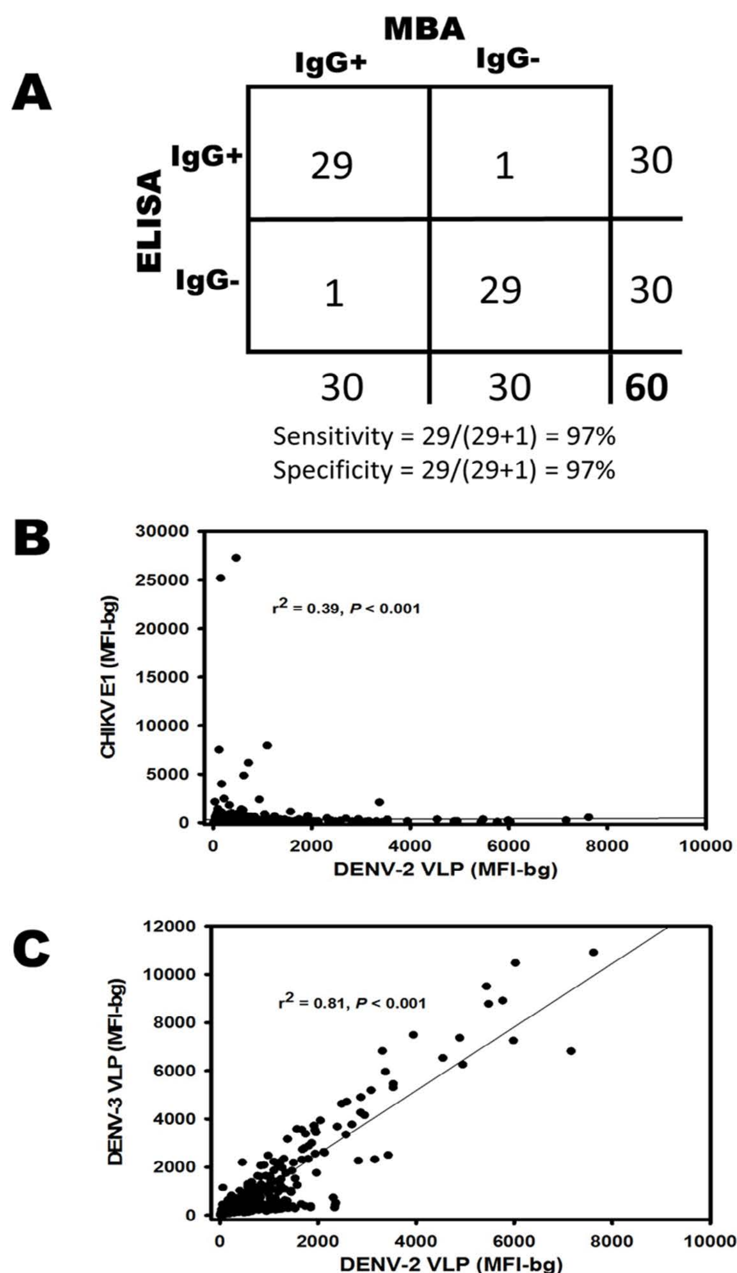

Figure 2: Comparison of DENV VLP MBA with "gold standard" ELISA by two-by-two table and correlations by scatter plots.

A) Two-by-two table comparing IgG detection by MBA with IgG detection by standard ELISA, resulting in an estimated sensitivity of 97% and a specificity of 97% by considering the ELISA assay the "gold standard" test; B) MBA scatter plot of MFI-bg (IgG responses) between DENV-2 VLP and CHIKV E1 (805 samples); C) MBA scatterplot of MFI-bg (IgG responses) between DENV-2 VLP and DENV-3 VLP (805 samples). Linear regression lines in B and C show correlation coefficients.

In contrast, using these same DENV VLP antigens, we showed that Haitian children produce increasing IgG responses to DENV with increasing age [21]. A Haitian child with longitudinal blood samples showed a primary DENV exposure with positive but low MFI-bg responses which decreased to negative responses before the child experienced a second DENV exposure that developed highly elevated IgG responses [21]. Likely, this child was exposed to DENV serotype 1 or 3, because the MFI-bg of DENV-3 VLP was higher in the primary and post anamnestic IgG responses than the MFI-bg of DENV-2 VLP [21]. In this study, all three antigens showed an increasing trend in median MFI-bg (IgG responses) from grades 1-6, which was significant for DENV-2 and CHIKV E1 ($p < 0.038$) and approached significance for DENV-3 ($p = 0.051$). Thus, the CHIKV and DENV data on these children, who may serve as a sentinel group, may provide useful information on school locations in need of *Aedes* mosquito control programs, such as those schools with red symbols (Figure 1A, Figure 1B, and Figure 1C).

Of 805 students assessed by MBA, 6.2, 24.3, 14.8% tested IgG positive to the CHIKV E1, DENV-2 VLP and DENV-3 VLP antigens, respectively. By a CDC standard method to measure IgG using a capture IgG ELISA, the CHIKV E1 coupled beads in the MBA were 90% sensitive and 85% specific, and here, the DENV-2 VLP and the DENV-3 VLP coupled beads in the MBA were 97% sensitive and 97% specific [21,25]. These validated beads

showed lower prevalence to CHIKV than to DENV, and this was not only consistent in a study in Mali but also in a study in the adjacent country of Burkina Faso where 76 German overseas workers spending a minimum of four months in Burkina Faso (1987-1993), were 1.3 and 9.2% positive for CHIKV and DENV, respectively [12,30]. It has been shown that the environmental and climatic conditions in the areas studied here, as well as in Burkina Faso, are suitable for mosquito breeding [31]. DENV outbreaks occurred in Burkina Faso in 1925, and 2013, and it was found that the DENV-2 serotype was the most prevalent [32,33]. Of interest, here, the DENV-2 VLP was higher in prevalence (24.3%) than the DENV-3 VLP (14.8%).

The prevalence of CHIKV and DENV shown here in Mali may seem relatively low, especially, when compared to countries like Haiti where children, ages 6-14 years, reached 70-80% prevalence in less than one year post CHIKV confirmation in Haiti and 70-90% prevalence for DENV [21]. Unlike Haiti, CHIKV has been endemic in Mali since 2009, well past the blood sample collection in this study [12]. However, despite the young age, some schools showed elevated prevalence of 21.1% for CHIKV E1 and 66.7% for both DENV-2 VLP and DENV-3 VLP, indicating areas of high mosquito transmission. Further, the prevalence of CHIKV and DENV is likely under-reported in many African countries, because of the heavy malaria burden and the lack of diagnostic technology to distinguish diseases causing febrile illness.

Sat Scan software found no significant cluster of CHIKVE1 prevalence, likely due to the relatively low prevalence of CHIKV in the students. In contrast, SatScan software found two significant clusters ($P < 0.001$) containing the same six schools for both DENV-2 VLP and DENV-3 VLP (Figure 1B and Figure 1C). Despite the wide distribution, unknown factors could be linking these six schools as a cluster for DENV-2 VLP and DENV-3 VLP (Figure 1B and Figure 1C) to make them significant (RR > 1.0) from the other schools, thus, providing potential information on areas of needed mosquito control. Of interest in a report on this same Mali study using the same DBS, these same six schools showed high prevalence of positive IgG responses to *Plasmodium falciparum* and *P. vivax*, which are transmitted by the *Anopheles* mosquito that has different biting habits compared with *Aedes* [18]. As such, these cluster analyses would benefit from more schools, especially in the northern part of the study area, and a more spatially-dense sample would provide more detailed information on the shape, and potentially number, of spatial clusters.

The MBA is an excellent serological technique that utilizes and collects data on multiple antigens simultaneously, thus, conserving specimens and lowering cost and labor. Cytokine and other analytic quantitation by MBA have shown sensitivities to be at least equal to ELISA [34-36]. Funding for single disease surveillance is limited, and the MBA here included 38 antigens from 22 different pathogens, and some have been reported [15-18]. The MBA is notable in that not only different researchers on various diseases can participate, as we did in this WASH in schools evaluation, but also can provide additional serological information on a study other than the intended primary pathogens.

Acknowledgements

We would like to thank the parents, students, and teachers who allowed us to conduct this work, as well as the Government of Mali. We would also like to thank the research team, including Abdoulaye Sow, Seydou Samaké, SalifIsmaïla Traoré, Fatoumata Habib Traoré, Karim Diamoutene, Yacouba Sogore, Alpha Oumar Haidara, as well as Niélé Hawa Diarra and Samba Diop from the University of Bamako. Thanks as well to the UNICEF, Water Aid, CARE, Oxfam, and Save the Children teams for their support, specifically Jérémie Toubkiss, Yagouba Diallo, Seydou Niafo, Touréba Keïta, Assitan Sogoré, Salimata Togola, Fatoumata Haïdara, Mamadou Diallo, Zoumana Cissé, Ousmane Haïdara, and Thierno Bocoum.

Disclaimer

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Service. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. The funder had no involvement in its design, in collection, analysis, or interpretation of the data or in the preparation of this manuscript.

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